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RESEARCH ARTICLE

Feeding through your gills and turning a toxicant into a resource: how the dogfish shark scavenges ammonia from its environment

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ABSTRACT

Nitrogen (N) appears to be a limiting dietary resource for elasmobranchs, required not only for protein growth but also for urea-based osmoregulation. Building on recent evidence that the toxicant ammonia can be taken up actively at the gills of the shark and made into the valuable osmolyte urea, we demonstrate that the uptake exhibits classic Michaelis–Menten saturation kinetics with an affinity constant (K_m) of 379 µmol l^{-1}, resulting in net N retention at environmentally realistic ammonia concentrations (100–400 µmol l^{-1}) and net N loss through stimulated urea-N excretion at higher levels. Ammonia-N uptake rate increased or decreased with alterations in seawater pH, but the changes were much less than predicted by the associated changes in seawater P_{NH_3}, and more closely paralleled changes in seawater NH_4^+ concentration. Ammonia-N uptake rate was insensitive to amiloride (0.1 mmol l^{-1}) or to a 10-fold elevation in seawater K^+ concentration (to 100 mmol l^{-1}), suggesting that the mechanism does not directly involve Na^+ or K^+ transporters, but was inhibited by blockade of glutamine synthetase, the enzyme that traps ammonia-N to fuel the ornithine–urea cycle. High seawater ammonia inhibited uptake of the ammonia analogue [^{14}C]methylamine. The results suggest that branchial ammonia-N uptake may significantly supplement dietary N intake, amounting to about 31% of the nitrogen acquired from the diet. They further indicate the involvement of Rh glycoproteins (ammonia channels), which are expressed in dogfish gills, in normal ammonia-N uptake and retention.

KEY WORDS: Elasmobranch, Urea, Ornithine–urea cycle, Rh proteins, Methylamine, Glutamine synthetase

INTRODUCTION

Ammonia is a ubiquitous toxicant in the aquatic environment, and is a major threat to fish (Randall and Tsui, 2002). Ammonia can exist in solution as either a gas (NH_3) or an ion (NH_4^+) depending on pH; here, we use the term ammonia to refer to their total, and the chemical symbols to refer to the particular species. Yet, most fish, such as the more than 30,000 teleosts, are ammoniotelic, producing ammonia as their major nitrogenous waste, and therefore must excrete it across the gills at the same rate at which it is produced so as to avoid the cerebral dysfunction and ultimate death that results from ammonia build up (Walsh et al., 2007). However, notable exceptions are the marine elasmobranchs (sharks, skates and rays), a relatively small (<1200 species) but ecologically very important group. These animals are ureotelic, producing urea, which is a much less toxic molecule, as their major nitrogenous waste (Wright, 1995). Furthermore, they are also ureosmotic, retaining high levels of urea in their body fluids so as to raise internal osmolality equal to or slightly higher than that of the external seawater, thereby avoiding the need for seawater drinking and its associated ionoregulatory costs (Smith, 1929, 1931; Kirschner, 1993). In these animals, metabolic ammonia is immediately trapped as glutamine, and then, through the ornithine–urea cycle, converted to urea, at the cost of 2.5 ATP per N put into urea (Shankar and Anderson, 1985; Campbell and Anderson, 1991; Kirschner, 1993; Ballantyne, 1997). Note that urea contains two atoms of N per molecule, so units of urea-N have been used in this study to allow direct comparison with ammonia-N.

The high levels of plasma urea (300–500 mmol l^{-1}=600–1000 mmol l^{-1} urea-N) necessitated by this ureosmotic strategy result in very large urea concentration gradients across the gills of elasmobranchs (Wright and Wood, 2016) because the external seawater typically has very low concentrations of urea (<0.1 mmol l^{-1}). There is clear evidence for the presence of both active and passive urea retention mechanisms at the gills, though the details remain controversial (Boylan, 1967; Wood et al., 1995, 2013; Pärt et al., 1998; Fines et al., 2001). Nevertheless, urea is still unavoidably lost to the external seawater across the branchial epithelium at a high rate, typically 400–600 µmol N kg^{-1} h^{-1}.

Thus, elasmobranchs need nitrogen not only for protein growth, like all animals, but also for the critical function of osmoregulation. Given the resulting high rates of urea-N loss across the gills and the opportunistic, irregular feeding habits of many of these predators (reviewed by Wood et al., 2007b), it is not surprising that they appear to be N limited in nature (Haywood, 1973; Wood et al., 2007a; Kajimura et al., 2006, 2008). Recently, based on experiments in which Pacific dogfish sharks (*Squalus acanthias suckleyi*) were exposed to a very high environmental ammonia concentration (1000 µmol l^{-1}), Nawata et al. (2015a) presented evidence that elasmobranchs may actively take up ammonia from the environment and use it to synthesize the essential osmolyte urea. The goals of the present study were to determine whether this phenomenon occurs at more environmentally realistic levels of external ammonia, to quantitatively assess whether it promotes net N retention and to characterize the mechanism(s) involved in terms of concentration dependence kinetics, sensitivity to pH and pharmacology.

MATERIALS AND METHODS

Experimental animals

Experiments were performed at Bamfield Marine Sciences Centre (BMSC) in September 2015 on adult male Pacific spiny dogfish (*Squalus acanthias suckleyi* Linnaeus 1758; mass 1.18–2.14 kg) under the guidelines of the Canada Council for Animal Care and with the approval of animal care committees at BMSC and the

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University of British Columbia (joint AUP A14-0251). The dogfish had been caught on hook and line by a commercial fisherman in nearby Barkley Sound under Fisheries and Oceans Canada collecting permit XR2392015. Prior to experimentation, the animals were held for approximately 3 weeks in a large (151,000 l) indoor concrete tank served with flowing seawater (12–13°C, 30 ppt salinity, dissolved O_2 >80% saturation). During holding, fish were fed twice a week with a 3% ration of dead hake (*Merluccius productus*), but were transferred to a smaller tank (1500 l) for at least 1 week of fasting before the start of experiments to ensure that they were in the post-absorptive state. Experiments were performed over the following 3 weeks during which fasting continued. Ammonia-N and urea-N excretion, as well as plasma concentrations of urea-N and ammonia-N, and osmolality all remain constant during 1–4 weeks of fasting in this species (Kajimura et al., 2008).

For all experiments, animals were transferred from the fasting tank to individual 40 l wooden boxes, fitted with a removable lid and coated with polyurethane, as described by Wood et al. (1995). Each box was served with perimeter aeration, and was fed with flow-through seawater. Additionally, the boxes were partially submerged in an external bath of flowing seawater so that the experimental temperature of 12.5±0.5°C could be maintained when flow to the boxes was suspended to allow flux measurements. Animals were allowed to settle in the boxes for at least 12 h before experiments were begun.

Experimental series

Series 1 – concentration dependence of ammonia-N uptake and urea-N excretion

Each dogfish (N=10) was exposed to elevated waterborne ammonia at nominal concentrations of 100, 200, 400, 800 and 1600 µmol l^{-1} for 10 h on different days. Actual waterborne ammonia and urea concentrations were measured at 2 h intervals, and therefore ammonia-N uptake and urea-N excretion rates could be calculated over five successive 2 h periods in each exposure. Additionally, each animal was exposed to a control condition, nominally zero ammonia; this exposure was limited to 6 h (three successive 2 h flux periods) to minimize any increase in ammonia concentration in the water. The six different test conditions were applied in randomized order to each animal, separated by a minimum of 14 h (overnight), during which the box was flushed with fresh seawater containing nominally zero ammonia.

At the start of each exposure, the water flow to the box was shut off while aeration continued, and the volume of seawater was set to 35 l. An appropriate volume of 1 mol l^{-1} NH_4HCO_3 (reagent grade, Fisher Scientific, Ottawa, ON, Canada) stock solution was then added and allowed to mix for 30 min, after which a 10 ml water sample was taken (0 h). Additional samples were taken at 2 h intervals up to 10 h, after which seawater flow was re-established, and the animal was allowed to stabilize overnight before the next ammonia concentration was presented. Blank tests demonstrated that there was no detectable loss of ammonia by volatilization, and that the addition of ammonia (as NH_4HCO_3) had no appreciable effect on seawater pH.

Series 2 – studies on the mechanism of ammonia-N uptake

In these experiments, various manipulations of the external seawater composition or of the animal itself were performed to investigate the possible mechanisms of ammonia uptake. Unless otherwise noted, an environmentally realistic ammonia concentration of 200 µmol l^{-1} (added as NH_4HCO_3) was used, and the box volumes were set to 35 l for these exposures. Ammonia-N fluxes were measured over 6 h, comprising three successive 2 h periods. With the exception of the methionine sulfoxamine (MSOX) trial (experiment v below), a paired design was used, employing six animals per test so that each fish served as its own internal control. The order of application of treatments was randomized, and there was a minimum of 18 h (overnight) between each treatment.

Experiment i: effect of seawater pH

The goal here was to greatly alter the concentration of NH_3, and therefore the partial pressure of NH_3 (P_{NH_3}) in the seawater without appreciably altering the concentration of NH_4^+. Dogfish were exposed to pH 7.95 (normal control seawater), pH 8.45 and pH 7.45 on different days. Thirty minutes prior to the start of each trial, the seawater ammonia concentration was raised to 200 µmol l^{-1} and the pH was set to the appropriate value using 0.1 mol l^{-1} NaOH or 0.1 mol l^{-1} HCl. Water pH was monitored at hourly intervals using a Symphony SP70P probe and meter (VWR, Radner, PA, USA) and adjusted as necessary to maintain pH within ±0.05 units of the target values.

Experiment ii: possible competition between ammonia and [^{14}C]methylamine

[^{14}C]Methylamine ([^{14}C]MA, $^{14}CH_3NH_2$) is a radiolabelled analogue of ammonia that is transported by Rhesus (Rh) glycoproteins in many mammalian (Nakhoul et al., 2010; Caner et al., 2015) and fish systems (Nakada et al., 2007, 2010; Nawata et al., 2007; Nawata et al., 2010a). In light of the recent discovery of Rh glycoprotein mRNA expression in the gills of *S. acanthias suckleyi* (Nawata et al., 2015a,b), the goal here was to determine whether ammonia would compete with [^{14}C]MA for uptake. Dogfish were exposed to [^{14}C]methylamine hydrochloride (specific activity 56 µCi µmol^{-1}; Moravek Biochemicals, Brea, CA, USA) at a concentration of 0.2 µCi l^{-1} (nominally 3.57 nmol l^{-1}methylamine) in the external seawater, in the presence of either zero or 1000 µmol l^{-1} ammonia (added as NH_4HCO_3). Blank tests demonstrated that there was no detectable loss of radioactivity by volatilization or adsorption to the boxes, so [^{14}C]MA uptake was monitored by the disappearance of radioactivity from the water, as sampled at 0, 2, 4 and 6 h.

Experiment iii: effect of amiloride on ammonia-N and [^{14}C]MA uptake

In light of debate on whether ammonia excretion is normally coupled to Na^+ uptake in the gills of marine elasmobranchs (reviewed by Wright and Wood, 2016), we employed the broad-spectrum Na^+ transport blocker amiloride (Benos, 1982; Kleyman and Cragoe, 1988) to evaluate whether the uptake of ammonia (and also of [^{14}C]MA) could be coupled to Na^+ transport. Amiloride hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was first dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), and then added to the seawater such that the final concentrations were 0.1 mmol l^{-1} amiloride and 0.1% DMSO. The control treatment was 0.1% DMSO alone. Additionally, in both trials the water also contained 200 µmol l^{-1} ammonia (added as NH_4HCO_3) and 0.2 µCi l^{-1} [^{14}C]MA (nominally 3.57 nmol l^{-1}methylamine).

Experiment iv: effect of high seawater K^+ concentration on ammonia-N uptake

NH_4^+ may substitute for K^+ through ionic mimicry in some biological transporters (Martinelle and Häggström, 1993; Good, 1988). The transporter H^+,K^+-ATPase, similar to that in the stomach (Smolka et al., 1994), has been identified in the gills of at

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least two elasmobranchs, including *Squalus acanthias* (Evans et al., 2004; Choe et al., 2004). In mammals, this transporter is known to readily transport NH_4^+ at its K^+ site (Codina et al., 1999; Cougnon et al., 1999). Therefore, the goal here was to examine the effect of a 10-fold elevation of seawater K^+ concentration to 100 mmol l^{-1} on ammonia uptake rate; this was achieved by addition of 90 mmol l^{-1} of reagent grade KCl (Fisher Scientific) to normal seawater, which contained a background K^+ concentration of 10 mmol l^{-1}. In the light of the limited amount of KCl available, a lower water volume (28 l) was used in these tests, and the control treatment was also run at this lower water volume. In both treatments, the water also contained 200 µmol l^{-1} ammonia (added as NH_4HCO_3).

In an additional test, the possible influence of the high KCl treatment on the transepithelial potential (TEP) across the gills was checked. Dogfish (N=5) were fitted with indwelling caudal artery catheters for TEP measurements, exactly as previously described (Nawata et al., 2015a). After overnight recovery of the animals in the standard wooden boxes, the TEP was measured first in seawater containing 200 µmol l^{-1} NH_4HCO_3, and then KCl was added to raise the K^+ concentration to 100 mmol l^{-1}, and the TEP was measured again after 3 h. A high impedance voltmeter (PHM 84 meter, Radiometer, Copenhagen, Denmark) was used to record TEP, using 3 mol l^{-1} KCl-agar bridges connected to Ag/AgCl electrodes (WPI, Sarasota, FL, USA). The measurement bridge was fitted to the arterial blood catheter and the reference bridge was placed in the water in the fish box. TEP values (mV) were expressed relative to the water side as 0 mV after correction for junction potential.

Experiment v: effect of MSOX on ammonia-N uptake

Trapping of ammonia by the enzyme glutamine synthetase is the first step in urea production in elasmobranchs (Shankar and Anderson, 1985; Kirschner, 1993; Wright and Wood, 2016). MSOX causes a slowly developing but irreversible inhibition of glutamine synthetase (Ronzio et al., 1969). Therefore, to test whether glutamine synthetase is involved in the uptake of ammonia from the environment, dogfish were injected intraperitoneally with either 60 mg kg^{-1} MSOX (Sigma-Aldrich) in 10 ml kg^{-1} of isotonic NaCl (500 mmol l^{-1}) or 10 ml kg^{-1} of isotonic NaCl alone. Different animals were used in the two treatments, and standard 6 h flux tests were run with 200 µmol l^{-1} ammonia (added as NH_4HCO_3) in the water. The MSOX dose (60 mg kg^{-1}) was approximately 10-fold greater than routinely used in teleosts (Sanderson et al., 2010; Zhang et al., 2013), but glutamine synthetase is more abundant in elasmobranchs (Kajimura et al., 2006). In a preliminary experiment with one animal, this dose proved not to cause mortality but did cause a slow decline in urea-N excretion rate after 48 h. Therefore, the 6 h tests were run at 35–41 h after injection, a time selected to avoid any secondary consequences due to internal urea declines. Urea-N excretion as well as ammonia-N uptake were measured in these trials.

Analytical techniques, calculations, and statistics

Ammonia-N (Verdouw et al., 1978) and urea-N concentrations (Rahmatullah and Boyde, 1980) in seawater were measured by colorimetric assays. In all cases, standard curves were constructed in the treatment water as some treatment water (e.g. that containing 0.1% DMSO) affected the slopes but not the linearity of the relationships. NH_4^+ and NH_3 concentrations as well as P_{NH_3} were calculated from ammonia and pH measurements using constants from Cameron and Heisler (1983).

Water samples (5 ml) for [^{14}C]MA analysis were immediately mixed with scintillation fluor (10 ml; Optiphase, PerkinElmer, Waltham, MA, USA), allowed to settle for 12 h to eliminate chemiluminescence, and then counted for beta-emissions on a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer). Tests showed that quench was constant.

Ammonia-N, urea-N and [^{14}C]MA flux rates (J_X) were calculated using the following equation:

$$J_X = [(X_1 - X_2) \times V]/(W \times T), \quad (1)$$

where X_1 and X_2 are the initial and final water concentrations, respectively, in any flux period, V is the volume of water in the fish box, W is the weight of the dogfish and T is the duration of the flux period. Positive values of J_X represent net uptake and negative values represent net excretion by the animal.

Sigma Plot version 10 (Systat Software, San Jose, CA, USA) was used to fit the Michaelis–Menten equation to the hyperbolic relationship between ammonia-N uptake and seawater ammonia-N concentration so as to derive the kinetic parameters J_{max} (maximum uptake rate) and K_m (inverse of affinity: the concentration of ammonia-N at which the uptake rate is 50% of J_{max}):

$$J_{Amm} = \frac{J_{max} \times [\text{Ammonia-N}]}{K_m + [\text{Ammonia-N}]}. \quad (2)$$

Data are expressed as means±1 s.e.m. (N=number of animals). In series 1, data were analysed by repeated measures ANOVA, followed by the Holm–Sidak *post hoc* test, while pair-wise comparisons were made by Student's paired two-tailed t-test. In series 2, experiments i–iv, data were analysed by Student's paired two-tailed t-test, with the Bonferroni correction applied in experiment i. In experiment v, Student's unpaired two-tailed t-test was used.

RESULTS

Series 1 – concentration dependence of ammonia-N uptake and urea-N excretion

Initial measured seawater ammonia-N concentrations (0 h) were close to the nominal values within each treatment, and thereafter tended to decline over the 10 h experimental period as ammonia-N was taken up by the dogfish. Ammonia-N uptake rates similarly tended to decline over time as the waterborne ammonia-N concentration decreased within each treatment, but uptake rates clearly increased as treatment ammonia-N concentration increased (Fig. S1). Uptake rates were greatest in the first three periods (0–2, 2–4 and 4–6 h) and, with the exception of the 800 µmol l^{-1} treatment, were not significantly different within a treatment at these times. Mean measured seawater ammonia-N concentrations over the first 6 h at nominal values of 100, 200, 400, 800 and 1600 µmol l^{-1} were 74.6±4.1, 155.1±8.1, 318.7±11.0, 625.8±35.2 and 1290.5±47.7 µmol l^{-1} (N=10). In the 6 h control experiment at a nominal zero ammonia-N concentration (mean measured concentration=5.9±1.8 µmol l^{-1}, N=10), ammonia was neither excreted nor taken up on a net basis (rate=−0.4±3.1 µmol ammonia-N kg^{-1} h^{-1}, N=10).

When the mean measured ammonia-N uptake rates (N=10) over the first 6 h were plotted against these measured seawater ammonia-N concentrations, the relationship was well described by the Michaelis–Menten equation (r^2=0.999, P<0.0001) with values of K_m=379±18 µmol l^{-1} and J_{max}=478±9 µmol ammonia-N kg^{-1} h^{-1} (Fig. 1A).

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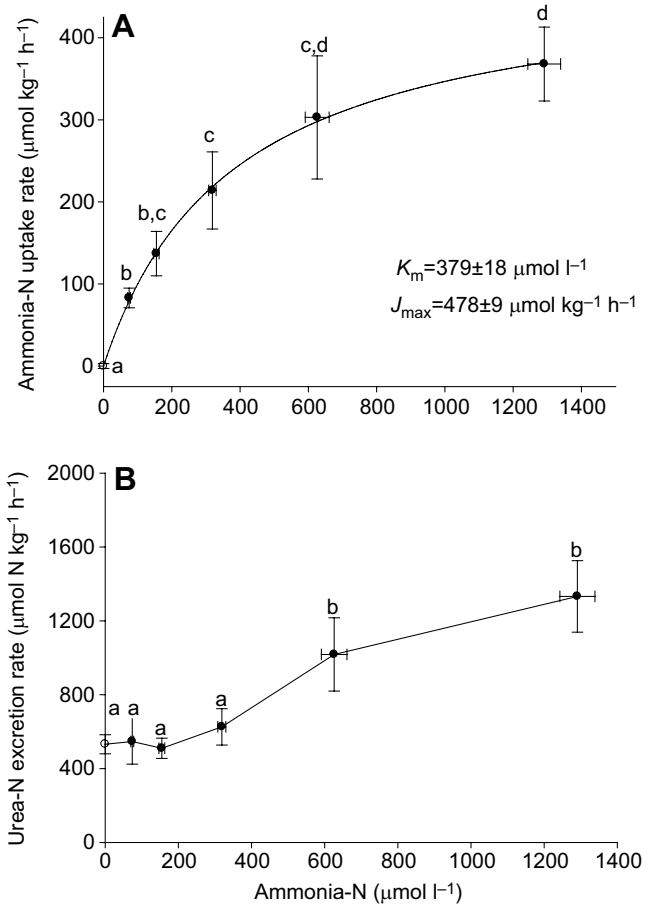

Fig. 1. Dependence of ammonia-N uptake and urea-N excretion rates on water ammonia-N concentration. (A) The relationship between the measured ammonia-N uptake rate averaged over hours 0–6 and the mean seawater ammonia-N concentration measured over that same period. (B) The relationship between the measured urea-N excretion rate averaged over hours 6–10 and the mean seawater ammonia-N concentration measured over hours 0–6 in the same exposures as shown in A. Means±1 s.e.m. (N=10). In A, the data were well described by the Michaelis–Menten equation (r^2=0.999, P<0.0001) with the K_m and J_{max} constants shown. Within a panel, means sharing the same letter are not significantly different (repeated measures ANOVA, followed by the Holm–Sidak test, P>0.05). From left to right, dogfish sharks were exposed to nominal starting ammonia-N concentrations of 100, 200, 400, 800 and 1600 μmol l^{-1}.

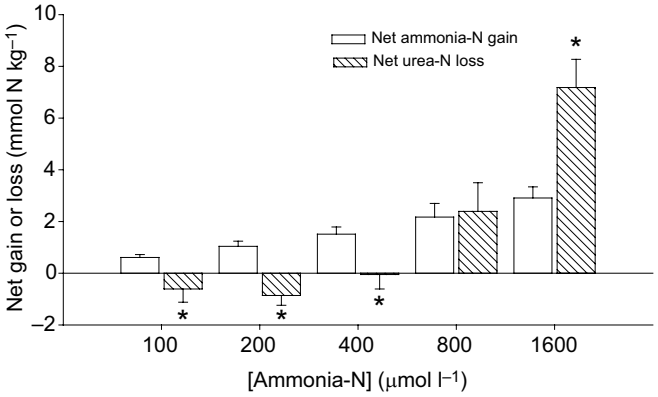

Fig. 2. Net ammonia-N uptake and urea-N loss over 10 h in dogfish sharks exposed to different starting ammonia-N concentrations. Means±1 s.e.m. (N=10). For this analysis, the measured baseline ammonia-N uptake and urea-N excretion rates at nominally zero ammonia-N in each animal have been subtracted. A negative urea-N loss represents a net urea-N gain relative to the baseline control. Asterisks indicate significant differences (Student's paired two-tailed t-test, P<0.05) between net ammonia-N uptake and net urea-N loss. Note that the dogfish were in positive N balance up to a starting ammonia-N concentration of 400 μmol l^{-1} and in negative N balance at 1600 μmol l^{-1}.

In the 6 h control experiment at a nominal zero ammonia-N concentration, the urea-N excretion rate was constant over time at 531.8±51.8 μmol urea-N kg^{-1} h^{-1} (N=10). Within each elevated ammonia-N treatment, urea-N excretion rates tended to increase over time, and to increase with treatment ammonia-N concentration (Fig. S2). Excretion rates were generally greatest and constant over the final three periods (4–6, 6–8 and 8–10 h) within each treatment, presumably reflecting the time delay necessary for the ornithine–urea cycle to convert the ammonia-N taken up into urea-N (Wood et al., 1995; Nawata et al., 2015a,b). When these mean 4–10 h urea-N excretion rates were plotted against the same measured seawater ammonia-N concentrations as in Fig. 1A, rates were approximately constant up to the nominal 400 μmol l^{-1} treatment, but increased significantly at the two highest treatments (Fig. 1B).

As the baseline ammonia-N and urea-N flux rates had been measured for each dogfish at a nominal zero ammonia-N concentration, it was possible to compare the net ammonia-N uptake over the entire 10 h period (i.e. baseline subtracted) with the net urea-N excretion (baseline subtracted) over the same period for each treatment (Fig. 2). This analysis demonstrated that at a nominal seawater ammonia-N concentration of 800 μmol l^{-1}, there was an approximate net N balance, and only at a nominal 1600 μmol l^{-1} was there a net N loss (−4.3 mmol N kg^{-1}). However, most importantly, at nominal seawater ammonia-N concentrations of 100, 200 and 400 μmol l^{-1} (i.e. the environmentally realistic range) over the whole 10 h period, net ammonia-N uptake significantly exceeded net urea-N excretion by 1.2–1.9 mmol N kg^{-1} (i.e. 120–190 μmol N kg^{-1} h^{-1}×10 h). At least in part, this occurred because urea-N excretion fell below baseline values.

Series 2 – studies on the mechanism of ammonia-N uptake

In these tests, a seawater ammonia-N concentration of 200 μmol l^{-1} was used not only for environmental relevance (see above) but also because it was in the steep part of the concentration kinetics curve (Fig. 1A), where experimentally induced increases or decreases in ammonia-N uptake rate would be most prominent.

Experiment i: effect of seawater pH

Raising or lowering seawater pH by 0.5 units from the control pH of 7.95 did not significantly change NH_4^+ concentration (variation=+1.5%, −18.0%; Fig. 3C) but greatly altered NH_3 concentration and therefore P_{NH_3} (variation=+229.5%, −73.4%; Fig. 3B). Therefore, the total variation in NH_3 concentration and P_{NH_3} over the 1.0 pH unit total range was 12.40-fold, while that in NH_4^+ concentration was only 1.24-fold.

Over this same 1.0 pH unit total range, ammonia-N uptake rates varied by only 2.08-fold (Fig. 3A), much closer to the variation in seawater NH_4^+ concentration (Fig. 3C) than that in seawater P_{NH_3} (Fig. 3B). As seawater pH was raised and lowered by 0.5 pH units from control, the respective variations in ammonia-N uptake rate were +47.5% and −29.2%. Only the difference in the rates between pH 8.45 and pH 7.45 was statistically significant (Fig. 3A).

This pattern of only modest sensitivity of ammonia-N uptake rate to large variations in seawater P_{NH_3} suggests that simple diffusive uptake of NH_3 across the dogfish gill is not very important, and that transport more closely follows the seawater concentration of NH_4^+.

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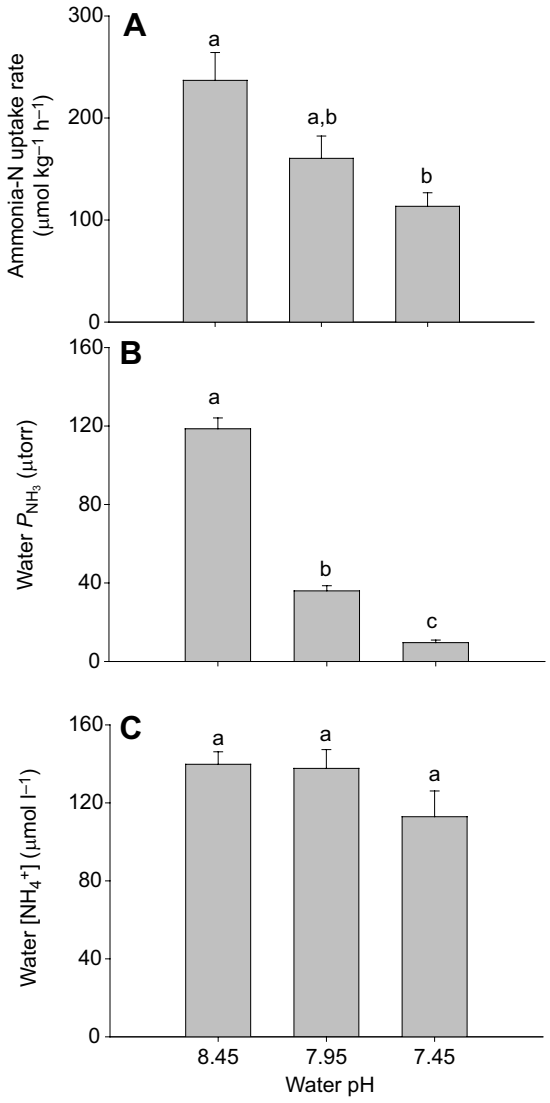

Fig. 3. Influence of seawater pH on ammonia-N uptake rate, water P_{NH_3} and water NH_4^+ concentration. Dogfish sharks were exposed over a 6 h period to a nominal starting seawater ammonia-N concentration of 200 μmol l^{-1}. (A) Ammonia-N uptake rate. (B) Water P_{NH_3}. (C) Water NH_4^+ concentration. Means±1 s.e.m. (*N*=6). Means sharing the same letter are not significantly different (Student's paired two-tailed *t*-test, with Bonferroni correction, *P*>0.05).

Experiment ii: possible competition between ammonia and [^{14}C]MA

Raising seawater ammonia-N concentration from 0 to 1000 μmol l^{-1}, which elevated the ammonia-N uptake rate from 0 to approximately 360 μmol kg^{-1} h^{-1} (Fig. 4A), resulted in a small but significant reduction (−17.5%) in the uptake rate of the ammonia analogue [^{14}C]MA (Fig. 4B). This result suggests that the two molecules interact in the transport system.

Experiment iii: effect of amiloride on ammonia-N and [^{14}C]MA uptake

As it was necessary to use 0.1% DMSO to keep 0.1 mmol l^{-1} amiloride in solution, we employed the same concentration of DMSO in the amiloride-free control; this had no effect on either ammonia-N or [^{14}C]MA uptake rates (data not shown). Our goal in this experiment was to test whether a Na^+-transport linked process, such as Na^+/NH_4^+ exchange, whose functional presence in elasmobranch gills remains controversial (reviewed by Wright and Wood, 2016) could be involved in ammonia-N and [^{14}C]MA uptake. Amiloride (0.1 mmol l^{-1}) had no effect on ammonia-N uptake (Fig. 4C), but reduced [^{14}C]MA uptake by 46%, a highly significant inhibition (Fig. 4D).

Experiment iv: effect of high seawater K^+ concentration on ammonia-N uptake

Raising the seawater K^+ concentration from 10 to 100 mmol l^{-1} had no effect on the ammonia-N uptake rate (Fig. 5A), suggesting that the uptake pathway is not shared with K^+. However, we were concerned that high K^+ might hyperpolarize the TEP such that the potential became more negative inside. If this were to occur, the net electrochemical gradient for NH_4^+ uptake at the gills would be increased, and this might mask any direct inhibitory effect of elevated K^+ on the ammonia rate. However, after 3 h in 100 mmol l^{-1} K^+, the TEP had actually become significantly more positive (+9.5±2.6 versus −1.7±1.0 mV, *N*=5), perhaps due to elevated K^+ entry across the gills, so if anything, this change should have reduced ammonia-N uptake.

Effect of MSOX on ammonia-N uptake

MSOX was used to block glutamine synthetase. We reasoned that this ammonia-trapping enzyme might act as the proximate 'sink' for ammonia uptake, and thereby serve as the rate-limiting step in the overall ammonia-N uptake pathway. As the MSOX treatment caused a significant 38% inhibition of ammonia-N uptake rate (Fig. 5B), this idea was supported. Notably, urea-N excretion rate was not affected (control=565±78 μmol kg^{-1} h^{-1} versus MSOX=621±80 μmol kg^{-1} h^{-1}, *N*=7), suggesting that internal urea-N stores had not declined at the time of these measurements.

DISCUSSION

Concentration dependence of ammonia-N uptake and urea-N excretion

Ammonia uptake was well described by the Michaelis–Menten equation with a K_m in the range of environmental relevance (Fig. 1A). This type of relationship generally indicates that transport is mediated by transporters or channels, with the K_m indicative of the normal concentration range within which the system evolved to operate. To our knowledge, only one study (Nawata et al., 2010a) has measured the ammonia-N K_m value for a Rh protein in fish (rainbow trout Rhcg2 expressed in *Xenopus* oocytes). Although this appears to normally function as an efflux transporter in the gills of trout (Nawata et al., 2007), it is in fact bidirectional with a K_m value of 550 μmol l^{-1} (Nawata et al., 2010a), quite close to the present value of 379 μmol l^{-1} for dogfish gill ammonia-N uptake.

At low environmental ammonia-N concentrations (nominally 100, 200 and 400 μmol l^{-1}), urea-N loss rates were less than ammonia-N uptake rates, so that the animal was able to make a net 'N profit' (Fig. 2). In part, this occurred because urea-N excretion rates dropped below baseline fasted values. Interestingly, in two previous studies on the same species, urea-N excretion rates also dropped below baseline fasted values following feeding, a response that was statistically significant in one study (Wood et al., 2007a) but non-significant in the other (Wood et al., 2005). This suggests that the slight rise in internal ammonia levels that occurs at this time (Wood et al., 2010) may serve as a signal to at least temporarily reduce the rate of branchial urea-N loss both in the presence of moderately elevated environmental ammonia-N concentrations as well as during the post-prandial period, thereby contributing to overall N conservation.

The net stimulation of urea-N excretion in excess of ammonia-N uptake (Fig. 2) at the highest seawater ammonia-N

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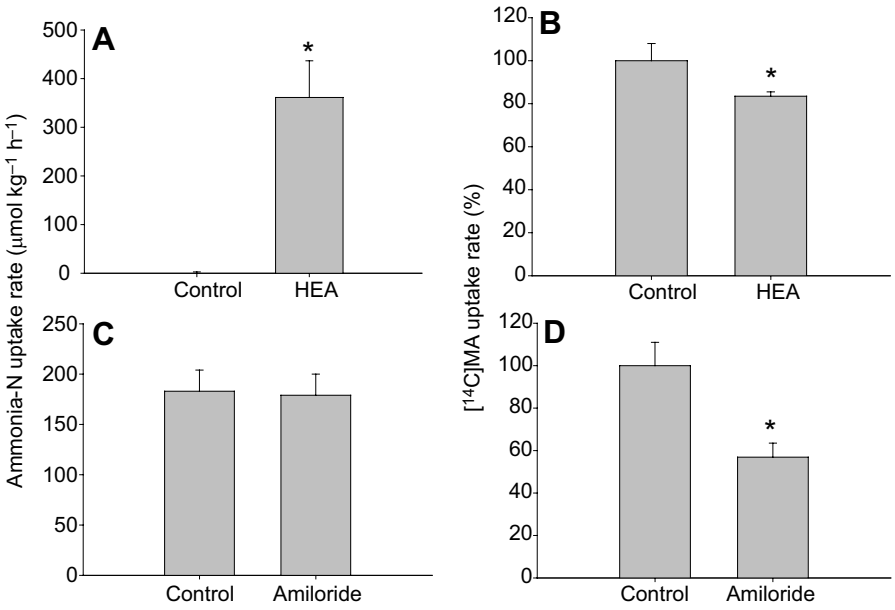

Fig. 4. Influence of high environmental ammonia-N and external amiloride on ammonia-N and [^{14}C] methylamine uptake rates. (A,B) Uptake rate of ammonia-N (A) and the ammonia analogue [^{14}C] methylamine ([^{14}C]MA; B) in dogfish sharks exposed over a 6 h period to high environmental ammonia-N (HEA, 1000 μmol l^{-1}) versus control conditions (no HEA). (C,D) Uptake rate of ammonia-N (C) and [^{14}C] MA (D) in the presence of external amiloride (0.1 mmol l^{-1}, in 0.1% DMSO) in dogfish sharks exposed over a 6 h period to a nominal starting ammonia-N concentration of 200 μmol l^{-1} versus control conditions (0.1% DMSO alone). Means± 1 s.e.m. (*N*=6). Asterisks indicate a significant difference (Student's paired two-tailed *t*-test, $P<0.05$) between experimental and control rates.

concentration (nominally1600 μmol l^{-1}) was previously reported in *S. acanthias suckleyi* exposed to 1000 μmol l^{-1} (Nawata et al., 2015a), and has also been seen in sharks infused directly with comparable ammonia-N loads (Wood et al., 1995; Nawata et al., 2015b). Very likely, it results from a stimulation of urea-N production by the ornithine–urea cycle in excess of the requirements for ureosmotic homeostasis, perhaps as a strategy to avoid ammonia intoxication (Walsh et al., 2007). Under these circumstances, the complex retention mechanisms that normally serve to minimize losses of urea-N at the gills (Boylan, 1967; Wood et al., 1995, 2013; Pärt et al., 1998; Fines et al., 2001) would be overwhelmed.

Environmental and physiological relevance of branchial ammonia-N uptake

In the open oceans, ammonia concentrations are in the low micromolar range, but may rise to or surpass the K_m for the uptake system identified here (379 μmol l^{-1}) in coastal and estuarine waters (Eddy, 2005). In the future, these levels are expected to increase as a result of non-point source runoff from agriculture (Howarth et al., 2002) and intensive aquaculture operations (Tovar et al., 2000). Furthermore, ammonia appears to function as a shark attractant (Mathewson and Hodgson, 1972; Gilbert, 1977). Water quality objectives for marine waters set by regulatory authorities are typically in the K_m range or higher (reviewed by Ip et al., 2001). Therefore, sharks may well exploit this system for ammonia-N uptake in nature.

Is ammonia uptake through the gills quantitatively important in terms of the shark's total N budget? The data of Kajimura et al. (2006) are useful to put this net N retention at the lower seawater ammonia-N concentrations into perspective. These workers concluded that the amount of N in a typical satiation meal (32 g of teleost fish) for a 1 kg dogfish would be about 52 mmol N, sufficient to satisfy branchial urea-N losses for about 4–5 days. Digestion takes about this long and the animal will not take another satiation meal for a comparable period (Wood et al., 2007b). Net N retention from ammonia-N uptake from seawater over that same time period (150 μmol N kg^{-1} h^{-1}×108 h) would provide a supplement of about 16.2 mmol N kg^{-1}, or 31% of the N acquired from the meal. Indeed, it would replace about 1.4 g of protein or 9 g of muscle that would otherwise be 'wasted' to replace branchial urea-N losses (Kajimura et al., 2008).

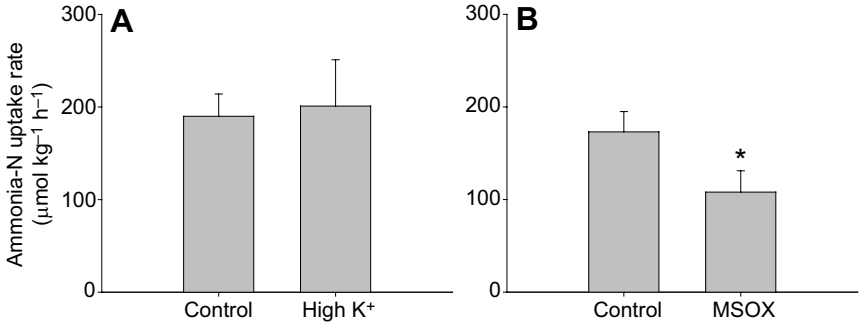

Fig. 5. Influence of high K^+ concentration and methionine sulfoxamine on ammonia-N uptake rate. (A) Uptake rate of ammonia-N in the presence of a high K^+ concentration (100 mmol l^{-1}, added as KCl) in the external seawater versus control conditions (normal seawater K^+ concentration of 10 mmol l^{-1}) in dogfish sharks exposed over a 6 h period to a nominal starting ammonia-N concentration of 200 μmol l^{-1}. (B) Uptake rate of ammonia-N following pre-treatment with the glutamine synthetase inhibitor methionine sulfoxamine (MSOX, 10 mg kg^{-1} in 10 ml kg^{-1} isotonic saline injected intraperitoneally) versus control conditions (isotonic saline injection alone) in dogfish sharks exposed to ammonia-N as detailed in A. Means±1 s.e.m. (*N*=6). Asterisks indicate a significant difference (Student's paired two-tailed *t*-test, $P<0.05$) between experimental and control rates.

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The mechanism of branchial ammonia-N uptake

As noted earlier, the Michaelis–Menten pattern of uptake (Fig. 1) suggests that the transport is mediated by transporters or channels. The fact that only small changes in ammonia-N uptake rate occurred in response to large variations in seawater P_{NH_3} and more closely paralleled variations in seawater NH_4^+ concentration (Fig. 3) indicates that simple diffusive uptake of NH_3 across the dogfish gill is not very important. The pattern is, however, consistent with a carrier-mediated system involving Rh proteins. Our current understanding is that these proteins actually bind NH_4^+ and not NH_3 at the entrance to the channel, but paradoxically facilitate the transport of NH_3 and not NH_4^+ through the channel (Nakhoul et al., 2010; Nawata et al., 2010a; Wright and Wood, 2009, 2012; Caner et al., 2015). The NH_4^+ is deprotonated at the channel entrance, and the NH_3 moving through the pore must be reprotonated at the other side. The external pH, and therefore the apparent P_{NH_3} gradient, can therefore modestly influence net transport by influencing H^+ availability for 'ammonia trapping'. For example, for various trout Rh proteins expressed in *Xenopus* oocytes, 1.0 pH unit changes in the external media caused 2- to 5-fold variation in the uptake rates of the ammonia analogue [^{14}C]MA, rather than the 10-fold differences expected from the change in concentration of the unprotonated form of [^{14}C]MA (Nawata et al., 2010a).

Raising the seawater ammonia concentration from zero to 1000 µmol l^{-1} caused a small (17.5%) but significant inhibition of the uptake rate of the ammonia analogue [^{14}C]MA (Fig. 4B). This result suggests that the two molecules share a common transport system, and is again consistent with a mechanism mediated by Rh proteins. For trout Rh proteins expressed in oocytes, concentrations of ammonia approximately 2-fold greater than those used here are needed to achieve 50% inhibition of [^{14}C]MA uptake (Nawata et al., 2010a), and in the only comparable study on an elasmobranch Rh protein, a 10-fold higher concentration was needed to cause 100% inhibition (Nakada et al., 2010). In our experiments, we wished to avoid such high concentrations as they would probably have been fatal to the intact animal.

Amiloride (0.1 mmol l^{-1}) had no effect on branchial ammonia-N uptake rate (Fig. 4C). As amiloride is a broad-spectrum antagonist of both Na^+ transporters and epithelial Na^+ channels (Benos, 1982; Kleyman and Cragoe, 1988), this result would seem to eliminate any direct role for a Na^+-coupled mechanism in the ammonia-N acquisition process. However, the 46% reduction of [^{14}C]MA transport by amiloride (Fig. 4D) was initially surprising. Nevertheless, it is actually consistent with recent data on mammalian Rh proteins indicating that this 'unnatural substrate' may be transported via the channel by a slightly different mechanism from that for ammonia, and that this mechanism is sensitive to amiloride (Nakhoul et al., 2010; Caner et al., 2015). Perhaps amiloride affects Na^+-linked intracellular pH regulation in epithelial cells, and this in turn affects [^{14}C]MA flux. The methylamine molecule is larger than the ammonia molecule, and its pK is more than 1 unit higher than the pK of ammonia.

A 10-fold elevation in seawater K^+ concentration had no effect on the rate of ammonia uptake (Fig. 5A). This indicates that ammonia is not taken up by a K^+ pathway such as K^+ channels or the H^+,K^+-ATPase transporter that has been identified in the gills of elasmobranchs (Evans et al., 2004; Choe et al., 2004). However, blockade of glutamine synthetase by MSOX resulted in a 38% inhibition of ammonia-N uptake rate (Fig. 5B). Glutamine synthetase is expressed in the gills of *S. acanthias suckleyi*, but also in a wide variety of other tissues (Chamberlin and Ballantyne, 1992; Steele et al., 2005; Kajimura et al., 2006), so it is unclear exactly where MSOX affected the ammonia-N uptake pathway. Nevertheless, we speculate that the key inhibition of importance occurred at the gills, because circulating glutamine-N levels are normally 6- to 8-fold greater than circulating ammonia-N levels in the blood plasma of comparably fasted dogfish (Wood et al., 2010). Regardless, this result does indicate that glutamine synthetase is at the downstream end of the uptake pathway, and that the normal fate of ammonia-N taken up at the gills is to be made into glutamine, which in turn would fuel the ornithine–urea cycle with nitrogen in tissues such as liver, intestine and muscle (Shankar and Anderson, 1985; Campbell and Anderson, 1991; Kirschner, 1993; Ballantyne, 1997).

Perspectives

The current data clearly demonstrate that *S. acanthias suckleyi* can harvest and retain a toxicant, ammonia, when it is present at environmentally realistic levels in seawater, and turn it into the valuable osmolyte urea. This finding adds to the growing body of evidence that fish can essentially feed through their gills by taking up N compounds from the external water. For example, rainbow trout (Wood, 2004), juvenile toadfish (Barimo and Walsh, 2005) and walleye (Madison et al., 2009) all grow more efficiently when environmental ammonia concentrations are moderately elevated, and hagfish take up amino acids from the external seawater across their gills and skin (Glover et al., 2011).

In the dogfish shark, the uptake system shows all the hallmarks of carrier mediation-saturable Michaelis–Menten kinetics, competitive actions with a similar substrate, and sensitivity to pharmacological blockade of a downstream receptor enzyme. The data of Nawata et al. (2015a) showing ammonia-N uptake against both P_{NH_3} diffusion gradients and NH_4^+ electrochemical gradients at the gills suggest that this is an active transport process. Under normal conditions, the effective permeability of the branchial epithelium to ammonia efflux is even lower than that to urea efflux (Wood et al., 1995), for which there is considerable evidence of active retention (Fines et al., 2001; Wood et al., 2013). We speculate that active ammonia uptake across the gills (i.e. 'ammonia-N scavenging') is a manifestation of a system that is normally used for active ammonia retention under control conditions.

The present evidence points to, but does not absolutely prove, the involvement of Rh proteins. The Rh transcripts expressed in the gills (Rhbg and Rhp2) are thought to be basolaterally located (Nawata et al., 2015a,b). Together with basolaterally located V-type H^+-ATPase (Tresguerres et al., 2005, 2006), these Rh proteins could function as a metabolon to pump ammonia-N from gill cells to blood, analogous to the apical Rhcg metabolon that pumps ammonia-N from gill cells to the external water in teleosts (Wright and Wood, 2009, 2012). In the kidney of another elasmobranch, *Triakis scyllium*, Nakada et al. (2010) have similarly proposed that basolateral Rhp2 serves to recover ammonia-N from the urine. Unfortunately, as yet, there are no pharmacological blockers of Rh proteins to test these ideas. However, there are both pharmacological and physiological tools available to manipulate the expression of V-type H^+-ATPase on the basolateral gill membranes of the dogfish shark (Tresguerres et al., 2005, 2006), providing an exciting pathway forward for future research.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.M.W. and M.G. jointly contributed to conceptual design, and jointly performed the experiments. C.M.W. wrote the manuscript and M.G. revised it.

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Supplementary information

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